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论著

机械加载通过促进髌下脂肪垫干细胞迁移及软骨分化治疗骨关节炎

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摘要 目的:探究机械加载对骨关节炎小鼠髌下脂肪垫干细胞(IFP-SCs)的动员作用。方法:54只雌性C57BL/6小鼠通过随机数字表法分为对照组(Sham组, $n=18$)、骨关节炎组(OA组, $n=18$)、骨关节炎机械加载组(OAL组, $n=18$)。采用DMM手术建立OA模型,OAL组进行2周的机械加载治疗(条件为1 N,5 Hz,6 min/d)。HE和番红O染色评估小鼠OA模型的软骨损伤程度。免疫荧光、细胞迁移实验、Western印迹等方法检测干细胞的迁移和软骨形成能力。结果:机械加载降低了OA小鼠的国际骨关节炎研究学会(OARSI)评分($t=4.025, P<0.01$)。与OA组相比,OAL组IFP-SCs的迁移能力增加($t=-5.142, P<0.001$),同时软骨中基质细胞衍生因子(SDF-1)和IFP-SCs中C-X-C趋化因子受体4(CXCR4)的表达增加($t=-4.403, P<0.01$)。此外,机械加载使软骨分化中重要的转录因子SRY相关高迁移率族盒蛋白9(SOX9)上调,促进了IFP-SCs软骨分化($t=-11.170, P<0.01$)。结论:机械加载通过促进IFP-SCs迁移和软骨分化,促进骨关节炎小鼠软骨修复。

关键词 机械加载;骨关节炎;髌下脂肪垫干细胞;迁移;软骨分化

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Mechanical loading treats osteoarthritis by promoting the migration and chondrogenic differentiation of infrapatellar fat pad stem cells

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Abstract Objective: To explore the effect of mechanical loading on the mobilization of infrapatellar fat pad stem cells (IFP-SCs) in osteoarthritis mice. **Methods:** Fifty-four female C57BL/6 mice were divided into three groups by random number table method: the sham control group (Sham, $n=18$), the osteoarthritis group (OA, $n=18$), and the osteoarthritis with mechanical loading group (OAL group, $n=18$). The OA model was established by DMM surgery. The OAL group received loading treatment for 2 weeks (the conditions are 1 N, 5 Hz, 6 min/d). The degree of cartilage damage in a mouse model of osteoarthritis was evaluated with HE and Safranin O staining. Immunofluorescence, cell migration assay, and Western blotting analysis were used to evaluate the migration and chondrogenic formation ability of stem cells. **Results:** Mechanical loading reduced the OARSI (Osteoarthritis Research Society International) score of OA mice ($t=4.025, P<0.01$). Compared with the OA group, the migration of IFP-SCs was enhanced ($t=-5.142, P<0.001$), and the expression of stromal cell derived factor-1 (SDF-1) in cartilage and C-X-C motif chemokine receptor 4 (CXCR4) in infrapatellar fat pad stem cells (IFP-SCs) were increased ($t=-4.403, P<0.01$). In addition, mechanical loading promoted IFP-SCs chondrogenic differentiation via up-regulation of SRY-related high mobility group-box 9 (SOX9) which was an important transcription factor in chondrogenic differentiation ($t=-11.170, P<0.01$). **Conclusion:** Mechanical loading facilitates cartilage repair in osteoarthritis mice by promoting the migration and chondrogenic differentiation of infrapatellar fat pad stem cells.

Key words mechanical loading; osteoarthritis; infrapatellar fat pad stem cells; migration; chondrogenic differentiation

骨关节炎(OA)是一种以关节软骨退行性病变为主要特征的慢性疾病^[1],它引起的关节疼痛、运动功能障碍,带来了沉重的社会负担^[2]。目前的药物治

疗(如非甾体抗炎药和阿片类药物)在延缓OA的同时也会导致严重的不良反应^[3]。近年来,间充质干细胞(MSCs)在关节软骨重建中的修复作用越来越受到重视^[4]。在关节内或关节周围的多个干细胞龛中存在各种具有迁移潜能的MSCs^[5]。在早期OA生物力学作用下,这些能够到达软骨浅层的关节内MSCs

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是软骨修复的关键细胞^[6]。

髌下脂肪垫(IFP)是一种位于关节囊内、滑膜外的脂肪组织^[7],其中含有增殖能力很强的MSCs^[8]。并且,胚胎起源决定了其拥有更强的软骨分化潜能^[9],这些MSCs可以迁移至软骨表面进行修复^[10]。机械加载是一种低频率、强度小、作用于膝关节等滑膜关节的温和机械刺激,能模拟人体主动运动的物理治疗手段^[11],可以通过抑制炎症、促进骨髓MSCs软骨分化,促进血管形成、骨形成,分别用于OA^[12]、骨质疏松^[13]、股骨头坏死^[14]的治疗。前期的工作已经发现机械加载通过OA小鼠的骨-软骨串扰来防止软骨退化^[15],但是机械加载对髌下脂肪垫干细胞(IFP-SCs)的动员作用尚不清楚。本实验采用手术横断小鼠膝关节内侧副韧带和切除内侧半月板建立OA模型,评估机械加载对IFP-SCs迁移和软骨分化的影响,为物理治疗OA和内源性软骨再生提供新的见解。

1 材料与方法

1.1 材料

1.1.1 实验动物 54只SPF级雌性C57BL/6小鼠,约14周龄,体重18~20g,由中国人民解放军军事医学科学院实验动物中心提供[生产许可证号:SCXK(京)2014-0013]。每笼5只小鼠,自由摄取饲料和水。所有实验根据天津医科大学实验动物管理规定进行,并经天津医科大学伦理委员会批准,审批号为[SYXK(津)2019-0004]。

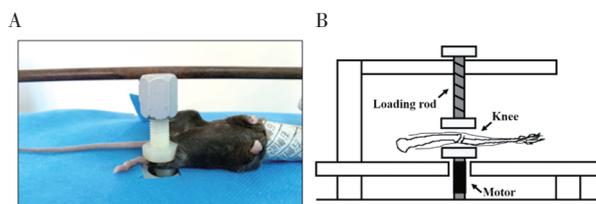
1.1.2 主要仪器和试剂 石蜡切片机(RM255)购自德国Leica公司。光学显微镜BX53购自日本Olympus公司。转化生长因子(TGF)- β 3购自中国Novoprotein公司。HE、番红O、Alcian Blue染料购自美国Sigma公司。胎牛血清、青霉素、链霉素和胰蛋白酶购自美国Invitrogen公司。抗体购自美国Abcam公司。

1.2 方法

1.2.1 动物分组及手术造模 54只小鼠采用随机数字表法分为对照组(Sham组, $n=18$)、OA组($n=18$)、骨关节炎机械加载组(OAL组, $n=18$)。用1.5%异氟烷麻醉小鼠,于右下肢膝关节内侧打开关节腔,使用手术显微镜和显微外科技术横断内侧副韧带,摘除内侧半月板。在右膝手术结束后,对左膝进行了同样的手术。Sham组只切开关节内侧皮肤。术后使用丁丙诺啡盐酸盐镇痛并使用恩氟沙星抗感染3d。

1.2.2 机械加载治疗 手术造模后第2天,OAL组接受机械加载。在机械加载期间,小鼠经1.5%异氟烷吸入麻醉,用定制的机械加载装备对双侧膝关节施加相同负荷(图1)。应用1N和5Hz的机械加载

治疗2周,每天6min(每侧腿各3min)。Sham组和OA组仅放置在加载装置上,没有实施加载力。加载后,允许小鼠在笼子中正常活动。



注:A:机械加载实物图;B:机械加载模式图

图1 机械加载示意图

Fig 1 Schematic diagram of mechanical loading for a mouse

1.2.3 组织学分析

1.2.3.1 组织处理:小鼠在手术后2周处死。剥离小鼠后腿,剔除皮肤、软组织及韧带,保留完整膝关节。将组织在10%中性福尔马林中固定2d后,在14%的EDTA中脱钙2周。将组织用石蜡包埋,冠状位切片,厚度为5 μ m。

1.2.3.2 HE、番红O/快绿染色:组织切片经二甲苯脱蜡、梯度乙醇水化后,进行HE和番红O/快绿染色,脱水透明后树脂封片,显微镜下观察组织病理形态学改变。使用Olympus CCD DP73软件进行相关数据的测量。软骨国际骨关节炎研究学会(OARSI)评分评估软骨损害程度。

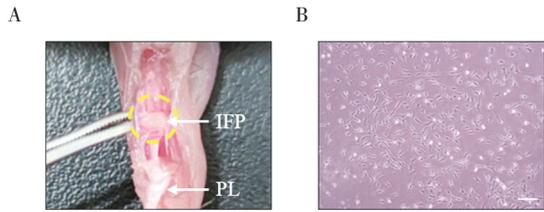
1.2.3.3 免疫荧光:组织石蜡切片用10%的山羊血清封闭,用一抗4 $^{\circ}$ C过夜孵育SDF-1(1:1 000),然后用荧光二抗染色。细胞核用DAPI染色。使用U-RFL-T荧光显微镜(Olympus, Tokyo, Japan)获得图像。计数每个样本中阳性染色细胞的数量。

1.2.4 细胞学实验

1.2.4.1 细胞分离培养:人道主义处死小鼠后,分离其后腿,切开发节囊上方皮肤,在髌韧带(PL)旁做切口,翻开PL,下方淡黄色组织即为IFP。用0.2% I型胶原酶震荡消化IFP 1h,用70 μ m细胞筛滤去多余脂肪组织。由于小鼠的IFP体积很小,为了得到足够数量的原代细胞,每组使用9只小鼠,将同一分组的3只小鼠种在一个孔板。IFP-SCs在添加100 U/mL青霉素、100 μ g/mL链霉素和10%胎牛血清(FBS)的DMEM中培养(图2)。

1.2.4.2 细胞迁移实验:Transwell(8 μ m,24孔板)用于细胞迁移实验。IFP-SCs悬浮在上室无血清的培养基中,下室含有含10%FBS的DMEM。共培养12h后,用福尔马林固定细胞,结晶紫染色通过孔膜底部的细胞以评估细胞迁移。

1.2.4.3 软骨诱导实验:IFP-SCs在软骨分化培养



注:A:IFP 的解剖部位;B:IFP-SCs 形态;IFP-SCs: 髌下脂肪垫干细胞;IFP:髌下脂肪垫;PL:髌韧带

图 2 IFP 的提取及光镜下 IFP-SCs 的形态(×100)

Fig 2 Extraction of IFP and the morphology of IFP-SCs under light microscopy(×100)

基中(MesenCult 增殖试剂盒补充 10^{-8} mol/L 地塞米松、50 μ g/mL 抗坏血酸、10 mmol/L β -甘油磷酸和 10 ng/mL TGF- β 3)培养 28 d。细胞在 10%缓冲福尔马林中固定,PBS 洗涤后,用 1%阿立新蓝染色,蓝染的细胞为软骨细胞。

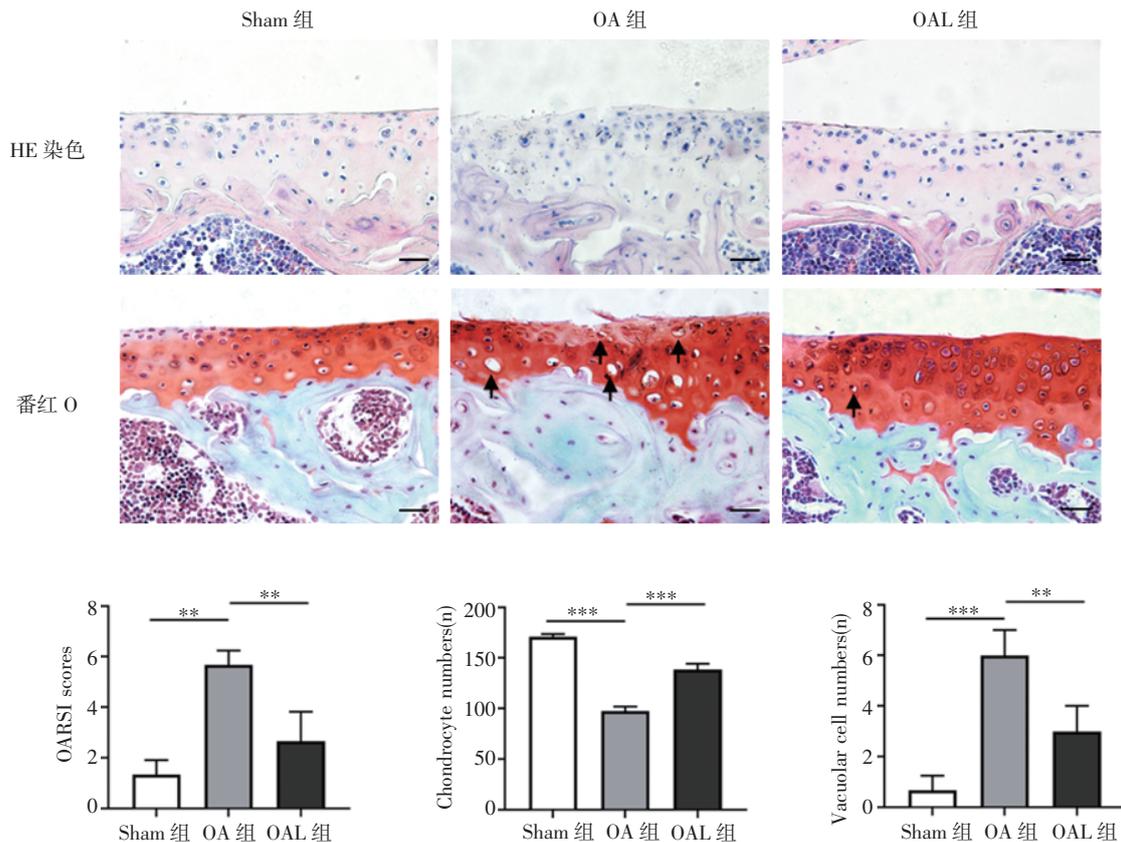
1.2.5 Western 印迹 IFP-SCs 在 PBS 中洗涤 3 次,在 RIPA 裂解缓冲液中提取总蛋白。在 4°C 下离心 10 min 去除细胞碎片。采用一抗 CXCR4(1:1 000)、SOX9(1:10 000)和 β -actin(1:10 000)在 4°C 过夜孵育。二抗室温(1:10 000)孵育 90 min 后,经化学发光后用凝胶成像系统检测相关蛋白的表达。

1.3 统计学处理 采用 SPSS 20.0 统计软件进行统计学分析。实验相关数据以 $\bar{x} \pm s$ 表示,多组间比较采用单因素方差分析(one-way ANOVA),组间多重比较行 LSD-t 检验, $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 机械加载对 OA 关节软骨的影响 HE 染色和番红 O 染色显示,Sham 组软骨表面光滑、完整,而 OA 组软骨表面粗糙、不平,OARSI 评分升高($t = -8.485, P < 0.001$)。经过机械加载治疗后,软骨表面平整,OARSI 评分降低($t = 4.025, P < 0.01$)。另外,与 Sham 组相比,OA 组的软骨细胞数量减低,空泡细胞数目增多,而机械加载使软骨细胞有所增加($t = 24.405, -9.605$,均 $P < 0.001$),空泡细胞有所减低($t = -8.000, -3.674$,均 $P < 0.01$,图 3)。

2.2 机械加载促进了归巢因子 SDF-1 的表达和 IFP-SCs 的迁移 免疫荧光结果显示,与 Sham 组相比,OA 组 SDF-1 表达有所增加,机械加载治疗后,OA 小鼠软骨中 SDF-1 的表达进一步增加($t = -8.148, -6.695$,均 $P < 0.001$,图 4)。同时,细胞迁移实验表明,与 Sham 组相比,OA 组的细胞迁移量增加($t = -5.085, P < 0.05$),OAL 组的细胞迁移水平最高

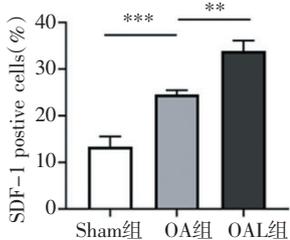
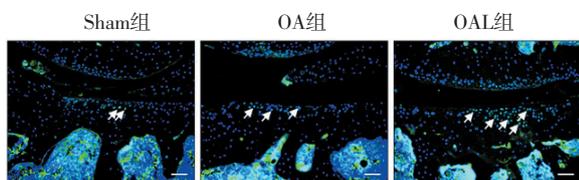


注:OARSI scores;国际骨关节炎研究协会评分; ** $P < 0.01$; *** $P < 0.001$; Sham 组:对照组;OA 组:骨关节炎组;OAL 组:骨关节炎机械加载组

图 3 内侧胫骨平台软骨的 HE 和番红 O 染色形态学检查(×400)

Fig 3 Morphological examination of the medial tibial plateau cartilage by HE and Safranin O staining(×400)

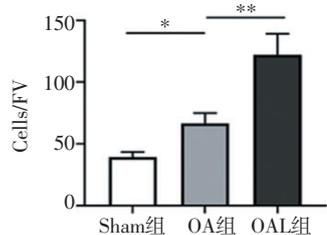
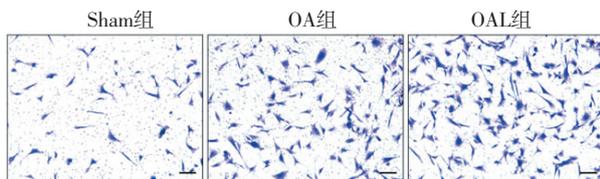
($t=-5.142, P<0.001$, 图 5)。进一步通过 Western 印迹检测迁移相关蛋白的水平。结果显示,与 Sham 组相比,OA 组 CXCR4 蛋白表达水平升高($t=-9.679, P<0.001$),机械加载进一步增强了其蛋白的表达水平($t=-4.403, P<0.01$, 图 6)。



注:SDF-1: 基质细胞衍生因子; * $P<0.01$; ** $P<0.001$; Sham 组: 对照组; OA 组: 骨关节炎组; OAL 组: 骨关节炎机械加载组

图 4 各组小鼠软骨中 SDF-1 的表达($\times 100$)

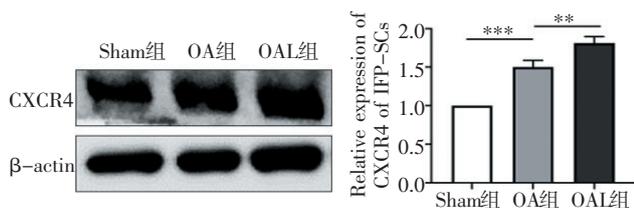
Fig 4 The expression of SDF-1 in cartilage of mice in each group ($\times 100$)



注:FV: 视野; * $P<0.05$; ** $P<0.01$; Sham 组: 对照组; OA 组: 骨关节炎组; OAL 组: 骨关节炎机械加载组; IFP-SCs: 髌下脂肪垫干细胞

图 5 各组 IFP-SCs 迁移($\times 100$)

Fig 5 Migration of IFP-SCs in each group($\times 100$)

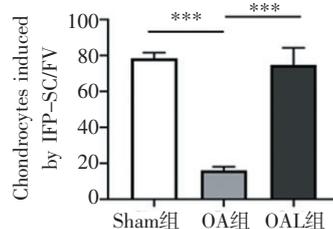
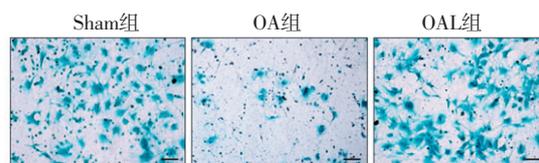


注:CXCR4:C-X-C 趋化因子受体 4; ** $P<0.01$; ** $P<0.001$; Sham 组: 对照组; OA 组: 骨关节炎组; OAL 组: 骨关节炎机械加载组

图 6 Western 印迹检测各组 CXCR4 表达

Fig 6 The expression of CXCR4 detected by Western blotting assay in each group

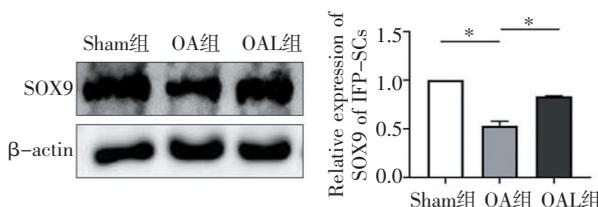
2.3 机械加载上调 SOX9 促进 IFP-SCs 软骨分化 通过软骨诱导实验,检测机械加载对 IFP-SCs 软骨分化的影响。与 Sham 组相比,OA 组中分化的软骨细胞数量明显减少。然而,OAL 组 IFP-SCs 分化为软骨细胞的数量显著增加($t=28.517, -10.462$, 均 $P<0.001$, 图 7)。为了进一步探究软骨分化的机制,采用 Western 印迹检测 IFP-SCs 中的 SOX9,与 Sham 组相比,OA 组的 SOX9 蛋白表达水平显著降低,而机械加载升高了 IFP-SCs 中 SOX9 蛋白表达水平($t=17.580, -11.170$, 均 $P<0.01$, 图 8)。



注:FV: 视野; ** $P<0.01$; Sham 组: 对照组; OA 组: 骨关节炎组; OAL 组: 骨关节炎机械加载组; IFP-SCs: 髌下脂肪垫干细胞

图 7 各组 IFP-SCs 诱导的软骨细胞($\times 100$)

Fig 7 Chondrocytes induced from IFP-SCs in each group($\times 100$)



注:SOX9:SRY 相关高迁移率族盒蛋白 9; * $P<0.05$; Sham 组: 对照组; OA 组: 骨关节炎组; OAL 组: 骨关节炎机械加载组

图 8 Western 印迹检测各组 SOX9 表达

Fig 8 The expression of SOX9 detected by Western blotting assay in each group

3 讨论

OA 是一种常见的关节软骨退行性疾病,由于肥胖、衰老等因素不断增加,其发病率也在逐年升高。因为关节软骨没有血管,一经损伤后软骨难以修复,随着疾病的进展,会引起关节疼痛、僵硬甚至运动障碍,严重影响生活质量。目前,OA 已成为重要的公共健康问题之一^[16]。因此,迫切需要开发 OA 早期的治疗办法以预防疾病进展。

早期药物治疗往往伴随一定的不良反应,相比于药物,运动训练更适合早期 OA。适度运动不仅能

降低体重过高等危险因素,更能预防 OA 的发生发展。机械加载作为一种可模拟主动运动的被动物理康复治疗方式,能够引起骨髓腔内压力的改变,驱动骨质内液体的流动,动员内源性干细胞,影响骨髓中的干细胞的分化、功能和归巢,调节骨髓内微环境^[1]。许多研究证明了机械加载对骨骼、关节的有益作用^[17-19]。其中包括机械加载通过增强自噬、促进骨髓干细胞软骨分化^[12]、抑制炎症反应^[20],以及抑制破骨细胞^[15]发挥作用,达到延缓 OA 进展的目的^[12]。本实验拓展了之前的研究范围,以关节内的脂肪组织为研究对象,探究机械加载条件下关节内干细胞的动员机制。笔者提取了 OA 小鼠的 IFP-SCs,证明了机械加载通过增强 IFP-SCs 迁移及软骨分化能力,延缓 OA 软骨破坏。OA 这种结果趋势与笔者预期一致,也与所报道的力学刺激对 OA 的有益作用相一致。本实验证明了膝关节机械加载这种力学刺激方式对关节内干细胞的动员作用,以及对 OA 关节软骨的保护及治疗作用。

IFP-SCs 在组织损伤后,干细胞的有效动员对其修复至关重要^[21]。研究表明,IFP-SCs 可以迁移到滑液中,修复受损的软骨^[5]。SDF-1 通过与其受体 CXCR4 发生反应,调节干细胞归巢到损伤部位^[22]。事实上,损伤后组织中趋化因子 SDF-1 浓度增加在短时间是有限的,在此期间,MSCs 虽然可以动员到损伤部位,但数量不足。研究发现,低强度机械刺激可以通过 SDF-1/CXCR4 轴,促进 MSCs 迁移^[23-24]。在本研究中,与 OA 组相比,机械加载增加了软骨表面 SDF-1 的表达。Western 印迹结果显示,机械加载增加了 IFP-SCs 中 CXCR4 的表达。细胞迁移实验显示,IFP-SCs 在机械加载后迁移能力增强。以上结果表明,机械加载可能通过 SDF-1/CXCR4 轴,促进 IFP-SCs 迁移,修复软骨。

另外,在软骨的形成和稳定性中,SOX9 是不可或缺的,特别是在 MSCs 的软骨分化中^[25-26]。文献表明,软骨修复失败的潜在机制之一是 OA 关节中的软骨细胞祖细胞和 MSCs 缺乏软骨生成转录因子 SOX9 的活性^[27]。本实验研究结果表明,在 OA 小鼠中 SOX9 的表达水平明显降低,而机械加载上调了 IFP-SCs 中 SOX9 的表达,并促进其软骨分化。这与低强度机械刺激可促进 IFP-SCs 分化为软骨细胞结果相一致^[28]。实验证明了机械加载作用下 SOX9 对 IFP-SCs 成软骨分化的贡献。

综上所述,本研究表明机械加载可以作为 OA 的一种新的治疗方法。机械加载可能通过激活 SDF-1/CXCR4 轴、促进 SOX9 表达,增强 IFP-SCs 的迁移和

软骨分化能力,发挥其治疗作用。但机械加载治疗下的迁移方式仍不明确,需进一步完善体内外研究进行验证,为临床治疗 OA 提供新的思路依据。

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